

Coevolution between harmful male genitalia and female resistance in seed beetles

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Reproductive conflict between the sexes is thought to be a key force in the evolution of many reproductive characters, but persuasive evidence for its significance is still scarce. The spectacular evolution of male genitalia that impose physical injury on females during mating has often been suggested to be a product of sexually antagonistic coevolution, but our understanding of these extraordinary adaptations is very limited, and there are no direct data addressing their evolutionary elaboration. We show that more spiny male genitalia causes more harm to females during copulation and provide comparative evidence for the correlated evolution between these antagonistic adaptations in males and a female counteradaptation (the amount of connective tissue in the copulatory duct) in a group of insects. By combining comparative and experimental methods, we demonstrate that imbalance of relative armament of the sexes affects evolution of the economics of reproduction: as males evolve genitalia that are more harmful relative to the level of female counteradaptation, costs associated with mating for females increase and population fitness is depressed. Our results unveil a coevolutionary arms race between the sexes and are consistent with a proposed link between sexual conflict, species' viability, and the risk of extinction.

extinction | sexual selection | speciation

Because males and females typically maximize their reproductive success in more or less different ways, the evolutionary interests of individuals of the two sexes may be quite divergent. Such sexual conflict is virtually ubiquitous and can lead to traits evolving in one sex that are beneficial for their bearers but are costly for individuals of the other sex (1). Coevolution between the sexes that is fuelled by sexual conflict is now recognized as a key evolutionary process that can shape both morphological traits and interactions between the sexes, depress population fitness, and affect rates of speciation and extinction (2–6). Theory suggests that common outcomes of such sexually antagonistic coevolution (SAC) are either polymorphism and frequency dependent selection or elaboration and coevolutionary escalation (1, 4, 7–9). The former is predicted primarily when the traits that males and females engage in sexual conflict show nominal variation (e.g., as in some signal-receptor systems) and the outcome of sexual interactions depends on how male and female traits match one another (10). The latter is predicted when male and female traits show ordinary quantitative variation, and the outcome of sexual interactions is instead determined by the level of elaboration of male and female traits (11).

Coevolutionary escalation can proceed much like an evolutionary arms race between the sexes (7), where the evolution of male persistence leads to the evolution of female resistance. Such evolutionary wars of attrition should leave their footprints in terms of the economics of reproduction (1), especially if costs of mating are dominated by those that result from sexual interactions rather than by those that derive from producing and/or bearing persistence and resistance traits (8). At any given point during a coevolutionary arms race, the outcome of male–female interactions and, ultimately, the direct costs of sexual interactions, should be affected by the balance of armament between males and females (2, 12). To unambiguously separate SAC from other intersexual coevolutionary processes, however,

it is necessary to demonstrate an effect of the relative expression of persistence and resistance on the direct costs of sexual interactions. Yet, no comparative study has been able to test for such effects (3, 12, 13).

Phylogenetic comparative methods remain the most powerful analytical tool for the direct study of coevolution (14). However, because comparative methods are correlational, it is difficult to distinguish causal biological relationships from spurious ones (15, 16). The integration of comparative methods with experimental studies offers a promising remedy for this limitation (17, 18) because experiments can shed unique light on causation. Here, we adopt such an integrative analytical strategy to study the evolution of male genitalia that cause harm to females and female responses to such evolution. Spiny male genitalia have evolved in many groups of animals (19–21) and can aid in male–male competition in several ways, for example by serving as an anchor during copulation, by allowing a more rapid passage of seminal products to females, or by overcoming female barriers to sperm (1). However, a pleiotropic side-effect of genital spines is harm to females (19, 22). This results in sexual conflict that should in theory select for female counteradaptations to minimize male-imposed harm (2, 3). The evolution of such counteradaptations could, in turn, promote the evolution of even more elaborate spines in males (1). Our understanding of these extraordinary adaptations, however, is limited because there are no data directly addressing their evolution.

Seed beetles (Coleoptera: Bruchidae) are an established model system for the study of reproductive conflict between the sexes: male genitalia are armed with sclerotized spines, and these spines penetrate the wall of the female copulatory duct during copulation and cause melanized scars in these tissues (19) [see supporting information (SI) Fig. 3]. Females suffer costs as a result of such injuries (19) but males do not benefit directly from harm inflicted on their mates (22). Instead, experimental evidence suggests that spiny genitalia benefit males by serving as an anchor during copulation (23). We secured morphological measures of the harmfulness of male genitalia (i.e., spinyess) and the robustness of the female copulatory duct (i.e., the proportion of the duct made up by connective tissue) in seven species of seed beetles, and performed experimental assays to quantify the cost of mating to females as well as female fitness. We then analyzed these data jointly in a phylogenetic comparative framework.

Results and Discussion

There is extensive variation across the species studied here both in the degree of elaboration of genital spines in males (Fig. 1)

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Abbreviations: SAC, sexually antagonistic coevolution; PGLS, phylogenetic generalized least squares regression.

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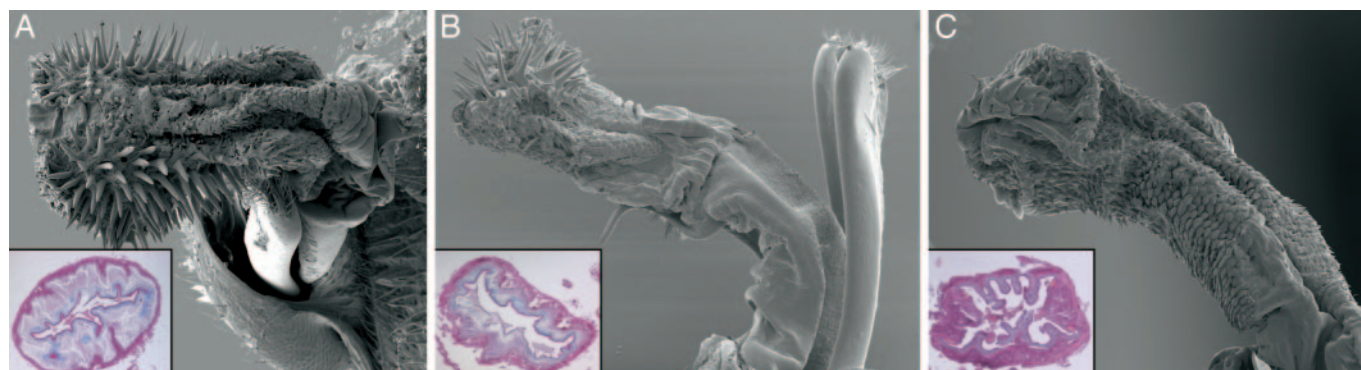


Fig. 1. The degree of elaboration of the genital spines that cause harm to the female reproductive tract during copulation varies markedly across seed beetle species, as does the extent to which the female copulatory tract is reinforced with connective tissue. To illustrate this variation, shown are scanning electron micrographs of male genitalia and cross-sections of the bursa copulatrix of females (connective tissue stained light blue and muscle tissue purple) of *C. analis* (A), *C. rhodesianus* (B), and *C. phaseoli* (C).

and in the cost of mating to females (24). Our experiments showed that even a single mating leaves traces of physical injury to females in several species (see SI Fig. 3), although the amount of scarring in the female copulatory duct increases with the number of matings performed (mating treatment: $F_{2,178} = 70.9$, $P < 0.001$) (25). Further, species differ both in the amount of scarring that occurs (species: $F_{6,178} = 58.3$, $P < 0.001$) and in how rapidly females accumulate scars over successive matings (interaction term: $F_{12,178} = 17.4$, $P < 0.001$).

If genital spines are involved in a coevolutionary arms race between the sexes, two key predictions can be made. First, the elaboration of spines should coevolve with resistance to spines in females. In agreement with this prediction, we found that the degree of harmfulness of male genitalia exhibited positive correlated evolution with reinforcement of the wall of the bursa copulatrix with connective tissue in females [phylogenetic gen-

eralized least squares regression (PGLS), $\beta = 0.13$, $P_{\text{dir}} = 0.015$; see Fig. 2]. Thus, as males evolve more harmful genitalia, females evolve a more robust copulatory tract.

Second, and more importantly, if coevolution between the sexes is balanced, then adaptation in one sex should be matched by counteradaptation in the other, and neither should thus be related to the economics of sexual interactions when analyzed separately. For example, the evolution of more harmful genitalia should not in itself be associated with more harm to females, simply because females should be more resistant to harm in taxa with more harmful male genitalia. In more general terms, SAC predicts that adaptation in one sex thus will be obscured by counteradaptation in the other, and SAC is notoriously difficult to unveil as a result of this coevolutionary coupling of the sexes (1, 26). One promising resolution of this dilemma is to analyze the effect of coevolutionary imbalance between male persistence and female resistance, by using either experimental evolution (2) or comparative methods (12). By using multivariate statistical models in a phylogenetic framework, one may test, for example, whether the evolution of more harmful genitalia is associated with more harm to females given that female resistance to harm is kept constant. When the independent effects of male persistence and female resistance are thus analyzed jointly, theory predicts that both should show effects on the outcome of sexual interactions (1, 12) (Fig. 2). As predicted, we found that neither the evolution of harmfulness of male genitalia nor the evolution of robustness of the female genital tract was significantly related to the cost of mating in females or to female fitness when analyzed separately in univariate regression models (Table 1). However, multiple regressions, assessing the independent effects of male persistence and female resistance, showed the predicted effects of both harmfulness of male genitalia and robustness of the female copulatory tract: the evolution of relatively harmful male genitalia, or equivalently relatively frail female tracts, was significantly associated not only with an increased cost of mating but also with a depression of our measure of female fitness across species (Table 1). Additional analyses showed that these key results are statistically robust (see SI Text for additional statistical analyses).

Our main results strongly support a role for SAC in the evolution of these remarkable characters. The scenario that emerges from our analyses is one where evolutionary modifications of male genital spines, driven by postmating sexual selection (21, 25, 27), imposes direct selection in females to alter their investment in counteradaptations to these spines. Although evolution is apparently balanced on average around this coevolutionary trajectory, minor imbalance in the relative armament

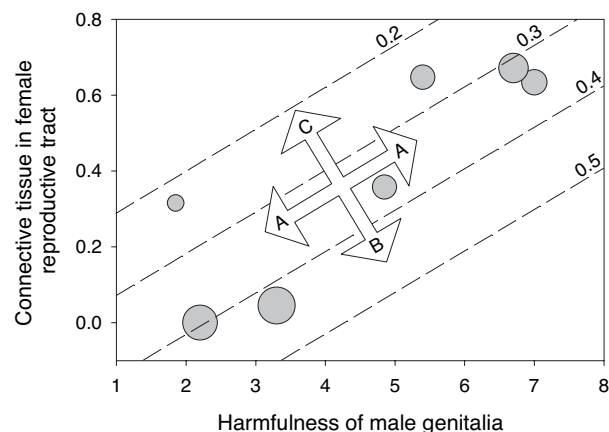


Fig. 2. The evolution of more harmful genitalia in males is correlated with the evolution of a more robust copulatory tract in females. Theory predicts that as species evolve up and down a balanced coevolutionary trajectory (A's in arrow), the economics of reproduction should be little affected. However, the evolution of relatively more harmful genitalia (B in arrow) should lead to an increased cost of mating and a depression of population fitness, whereas the evolution of better defended females (C in arrow) should result in the reverse. Shown is an equal height contour plot, where lines represent the cost of mating predicted from a multiple regression of species-level data with cost of mating as dependent variable and harmfulness of male genitalia and the proportion of connective tissue in the female reproductive tract as independent variables. Symbols represent species values, and the size of the symbols is proportional to the cost of mating for each species (see Table 1 and SI Table 3 for statistical evaluations).

Table 1. Univariate and multiple phylogenetic generalized least-squares regressions of the effects of harmfulness of male genitalia and the amount of connective tissue in the copulatory tract of females

Regressions	Cost of mating			Lifetime offspring production			Scarring of female copulatory tract		
	β	SE	P	β	SE	P	β	SE	P
Univariate regressions									
Harmfulness	−0.004	0.003	0.887	−1.388	3.462	0.705	0.757	0.226	0.021
Connective tissue	−0.200	0.135	0.197	1.483	2.047	0.501	5.203	0.891	0.002
Multiple regression									
Harmfulness*	0.007	0.003	0.031	−10.29	4.025	0.039	0.182	0.269	0.268
Connective tissue*	−0.549	0.153	0.015	6.641	2.462	0.034	4.289	1.647	0.149

*Directed tests (P_{dir}) reported; see text for predictions.

of the sexes at any given point affects the cost of mating to females and female fitness.

The amount of scarring in the female reproductive tract that results from mating showed a somewhat different evolutionary pattern. In contrast to the other dependent variables, the degree of harmfulness of male genitalia and the amount of connective tissue in the female copulatory tract were both positively related to the amount of scarring when analyzed separately, and the multiple regression model suggested that amount of scarring did not decrease with a more robust copulatory tract (Table 1 and SI Table 3). Notably, the amount of scarring suffered by females did not show correlated evolution with the cost of mating (PGLS, $\beta = -0.016$, $P = 0.618$), suggesting that scarring *per se* is a poor measure of the net cost of mating across species. One possible explanation for this result is that females have evolved additional resistance adaptations, besides a reinforced copulatory tract, to better cope with physical injury during copulation. It has been suggested that increased female investment in immunocapacity represents a female resistance adaptation to trauma caused by injurious male genitalia during copulation in other insects (28). If this is true also in seed beetles, the evolution of a more robust female copulatory tract may be positively associated with an immune system better adapted to cope with injury and scarring, which could generate the pattern of correlated evolution observed here. In effect, genital injury and scarring then would tend to be less costly in taxa where females also exhibit a more robust copulatory tract. Theory suggests that different forms of female resistance adaptations, such as structural and physiological, may indeed exhibit correlated evolution (1).

Because sexual conflict involves direct selection, it has been suggested repeatedly that SAC has the potential to critically affect population fitness (27) and ultimately even the risk of species extinction (1, 3, 9). Although it is unclear to what extent our measure of population fitness reflects the risk of population extinction, our data are at least consistent with this tenet. Evolution of an increased cost of mating to females was associated with evolution of depressed female fitness in these beetles (PGLS, $\beta = -102.8$, $P_{dir} = 0.026$). Further, the relationship between evolution of male persistence and female resistance on one hand and the economics of reproduction on the other was rather strong. The R^2 values in the PGLS multiple regression models, by using harmfulness of genitalia and the amount of connective tissue as the sole explanatory variables, were 0.76 for cost of mating and 0.65 for lifetime offspring production. We note that these values are analogous to R^2 values from regressions using phylogenetically independent contrasts forced through the origin (29). The corresponding R^2 values from conventional regressions using species level data were 0.72 and 0.18 (SI Table 3). Our analyses thus confirm that the economics of reproduction in this group partly reflects the degree of mismatch between male persistence and female resistance in SAC.

Although the origin of harmful male genitalia is no doubt the result of reproductive competition between males, as are other male traits that are costly to females (e.g., refs. 1, 21, 30, and 31), SAC apparently plays a key role in the subsequent coevolutionary dynamic of male and female traits in seed beetles. In general, male genitalia evolve very rapidly and divergently in animals (21, 32), and seed beetles are no exception to this rule. Many species in this group can only be reliably identified by the anatomy of male genitalia. Although it is clear that postmating sexual selection is responsible for genital evolution (33), no previous work has been able to identify the process by which such selection is generated (21). Our results strongly suggest that, in this group of beetles, sexual conflict and the resulting SAC can affect the evolutionary elaboration of intromittent genital traits in males by selection in females to minimize direct costs imposed by males.

There has been some discussion regarding the general importance of SAC for the evolution of reproductive traits (21, 33), and the resolution of this debate is complicated by the fact that it is very difficult to study SAC empirically. Our work suggests that analyses of the degree of mismatch between the sexes in adaptations that are involved in SAC can help reveal the underlying coevolutionary process in future work in this area. Although this requires both the successful identification and quantification of key traits in both sexes and experimental assays of the outcome of sexual interactions, we believe that important insights can be gained by this empirical strategy (2, 12, 13).

Materials and Methods

We studied seven closely related, and ecologically very similar, species of seed beetles that share a common mating system (i.e., polyandry) (24, 34): *Callosobruchus maculatus*, *C. subinnotatus*, *C. analis*, *C. rhodesianus*, *C. chinensis*, *C. phaseoli*, and *Zabrotes subfasciatus*. Beetles were kept under controlled conditions in the laboratory, at 27°C and 45 ± 10% relative humidity (see ref. 24 for methods used for maintenance and rearing).

Male Genitalia. Male genitalia evolve rapidly in this clade, and comparison across species is complicated by the fact that many species-specific genital traits lack homologies in other species within the group. Yet, an assessment of coevolution between males and females requires quantification of harmfulness of male genitalia that is independent of actual harm caused. We used two distinct approaches to secure such measures.

First, we traced outlines of male genitalia of all species (scanning electronic micrographs of lateral projections) and analyzed shape variation across species by means of elliptic Fourier analysis (32). Briefly, this method fits a nonlinear function to each outline and quantifies shape variation across objects as variance in the coefficients of the fitted function. This analysis was performed by using 50 harmonics in Morpheus et al. software (35), and the resultant matrix of 200 elliptic Fourier coefficients was subjected to a principal component analysis,

based on the covariance matrix. The first principal component from this analysis (EFA1) explained 41% of total variance in genital shape and described the ruggedness of male genitalia. We then recorded the number genital spines present in each species and combined this number with EFA1 score (by means of a conventional principal component analysis) to form an integrative morphometric measure (PC1) of putative harmfulness of male genitalia for each species.

Second, we presented a set of scanning electron micrographs of the genitalia of each species for 20 persons who were asked to rank the seven species according to harmfulness of the genital structures. The persons ranking genitalia, all of whom were professional biologists naïve to this particular taxa and problem, were given a standardized written instruction stating only that male genitalia cause internal injury to females during copulation in these beetles. The repeatability of single rank scores was $R_1 = 0.65$ [one-way analysis of variance (ANOVA): $F_{6,133} = 38.7$, $P < 0.001$]. The repeatability of the average rank score for each species, which forms our second measure of putative harmfulness of genitalia, was $R_{20} = 0.97$.

The two measures of harmfulness of male genitalia were highly correlated across species (Pearson product moment correlation $r_p = 0.96$, $P < 0.001$; Spearman rank correlation $r_s = 1.0$, $P < 0.001$). Further, analyses based on the two alternative measures yielded quantitatively very similar and qualitatively identical results: in no case did our ability or inability to reject null hypotheses differ depending on which measure was used. To restrict the length of our contribution, we thus only report analyses based on the second of the two measures of harmfulness of male genitalia in this article.

Female Copulatory Tract. The walls of the bursa copulatrix of females are made up largely by muscle tissue and connective tissue. Muscles allow contraction of the wall, and connective tissue adds tensile strength and resilience to the wall. We dissected out the bursa copulatrix of virgin females of all species ($n = 3$ or 4 females per species) and standard microscope preparations were made of the region where male genital spines reside during copulation (immediately above the entrance of the common oviduct and spermathecal duct into the bursa) (see Fig. 1). By using digital image analysis of cross-section preparations, we then measured the proportion of the area of the wall that was made up by connective tissue. Species differed significantly in this proportion (one-way ANOVA: $F_{6,17} = 27.8$, $P < 0.001$), and average proportion for each species was used as a measure of the robustness of the copulatory duct in subsequent comparative analyses.

Cost of Mating. Female bruchid beetles lay the majority of their eggs during the first few days of their adult life under standard laboratory conditions such as those used here (34), and the cost of mating is therefore manifested more as a reduction in lifespan than a reduction in lifetime egg production (24). Thus, we used a measure of the cost of mating in each species that represents the average reduction in lifespan suffered by multiply mated females compared with females mated only once. We randomly assigned females to either of two experimental treatments ($n = 15$ females per species and treatment): females were either mated multiply with several males or were mated only once with a single virgin male [see ref. 24 for a detailed account of the methods used (treatments C and D)]. We note that the measure of the cost of mating used here correlates closely with an alternative measure, where the effect of individual variation in egg production has been partialled out ($r_p = 0.98$, $P < 0.001$; $r_s = 1.0$, $P < 0.001$), and analogue analyses using these two measures yielded quantitatively very similar and qualitatively identical results. Species differed significantly in the cost of mating [test of the interaction term

between mating status (singly or multiply mated) and species ($F_{5,162} = 2.63$, $P = 0.026$), from a full two-way factorial analysis of covariance of variation in female lifespan where the number of eggs laid was included as a continuous covariate (see also ref. 24)].

Female Fitness. To quantify variation in female fitness across species, virgin females ($n = 20$ per species) were each mated with a virgin male and placed individually in chambers containing a superabundance of food resources (100 beans of the preferred host species per female; see ref. 24 for rearing conditions). Females were allowed to oviposit until their natural death, and our measure thus reflects potential reproductive rate. The number of offspring produced was subsequently recorded for each female. Variation across species in female size and lifespan, as well as in the amount of water/nutrition transferred by males at mating (36), affected offspring production. To yield a measure of female fitness that better reflects the costs of reproductive interactions between the sexes (including costs of investment in resistance traits) and that is comparable across species, we calculated a rate-sensitive measure of female fitness (37) by accounting for variation in these factors across species. We performed a multiple regression of species means using lifetime offspring production as the dependent variable, and female weight, female lifespan, and the weight of male ejaculate as independent variables. Residual lifetime offspring production from this model was then used as a measure of female fitness. Species differed significantly in female fitness (one-way ANOVA: $F_{6,125} = 38.1$, $P < 0.001$; see also ref. 24).

Scarring. We quantified the amount of scarring in virgin females, females mated once only, and females mated multiply ($n = 10$ females of each mating treatment and species) by first dissecting out the bursa copulatrix (SI Fig. 3) and then using image analysis to record the following variables for each female: the number of melanized scars, the total area of all scars, and the area of the region of the bursa that carried scars. No virgin females showed scarring in the bursa. The three measures of scarring were highly correlated, and we thus used the first principal component (explaining 74% of the variance across species in total scarring among single and multiple mated females) as an integrative measure of the amount of scarring. We tested for differences across species and for an effect of the number of matings, by using a full factorial two-way ANOVA of the amount of scarring where species and mating treatment were our factors (see first paragraph of *Results and Discussion*).

Comparative Methods. We first estimated the amount of phylogenetic signal present in our data by using Physig.m (38) and a well supported reconstruction of the phylogeny of this group based on molecular data (39) (see SI Fig. 4). This analysis revealed a sizeable phylogenetic signal (spinyess of male genitalia, $K = 0.76$; proportion of connective tissue in the female copulatory tract, $K = 0.43$; cost of mating, $K = 0.39$; female fitness, $K = 0.32$; amount of scarring, $K = 0.33$), suggesting that phylogenetic comparative analyses should be used. We thus used PGLS (14), as implemented in Regression.m (38), to assess evolutionary covariation between traits. The cost of mating, female fitness, or scarring to the reproductive tract was treated as the dependent variable, and harmfulness of the male genitalia and the amount of connective tissue in the female copulatory tract were independent variables. Analogous models of species-level data (SI Table 2) yielded results very similar to those of the PGLS models given here (SI Text and SI Table 3). When testing hypotheses in which the sign of the effect was predicted *a priori*,

we used directed tests (40). Directed tests enable detection of patterns that are opposite to predictions while retaining much of the statistical power of one-tailed tests. In all directed tests (denoted P_{dir}), we followed the convention of setting $\gamma/\alpha = 0.8$ (40).

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